Gas Chromatography

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The main mass of organic carbon in the aquatic environment and in soils/sediments is located in humic organic matter (HOM). A typical agricultural soil contains about 2–7% HOM in the upper level. Depending on the type of water (freshwater lake, marine origin, bog water, etc.), the content of aquatic HOM may range from 50 to almost 100% of the dissolved organic carbon. Terrestrial HOM acts mainly as a pollutant sink; aquatic HOM has a distinctive vehicle function. Thus, HOM influences fate, bioavailability and transport behaviour of organic and inorganic pollutants.

The detailed structural characterization of the ubiquitous HOM is an ambitious task because these polymers do not possess the uniform structure observed with other natural polymers. The extreme heterogeneity of both soil-derived and aquatic HOM in terms of chemical composition, monomer unit sequence, molar mass and functionality is associated with their genesis: for example, soil organic matter is composed of a variety of plant and animal residues in different stages of decomposition, of (radical) metabolites possessing mesomeric forms, and of products formed from these breakdown products. During the genesis, the chemical identity of the precursors is lost by abiotic and/or biotic condensation reactions.

The combined application of powerful analytical techniques may reveal some basic principles in HOM structure, but will probably never give a complete structure. Therefore, HOM analysis should not be targeted at the elucidation of discrete HOM structure but to recognizing substructures and functional groups (including their linkages), on the basis of which the fundamental interactions of HOM with environmental contaminants of organic and inorganic origin may be better understood. Nondestructive nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR) have proved to be very useful in providing information on functional groups and on the neighbourhood of substructures. Small scale analytical pyrolysis and controlled wet chemical degradation, in which the polymeric network is broken down to smaller subunits, constitute efficient supplements to nondestructive spectroscopic methods, in which an averaged structure is studied. Thermal and wet chemical degradation can give volatile products amenable to gas chromatography (GC) analysis, including hyphenated techniques, such as gas chromatography–mass spectrometry (GC-MS) and gas chromatography–atomic emission detection (GC–AED). The combination of data obtained from the structure-related mass spectrum and the element-related atomic spectrum is especially useful for the detection of heterocyclic compounds as well as chlorinated structures in humic-like chlorolignins. The basic assumption in this strategy is to correlate the destructive products with original moieties present in the starting HOM network.

In addition to structure-related analytical investigations, destructive methods can also be applied for elucidation of the carbon cycle in soils and water, for solving geochemical problems (such as the diagenesis of HOM), in recognizing pathways of emissions from biomass combustion, in revealing pesticide contamination of soils, in studying humification processes taking place in landfills, in studying chlorinated aqueous HOM, in screening residues in bioremediated soils, and for other applications.

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Conventional Analytical Pyrolysis

The traditional analytical pyrolysis, which can easily be performed using a flash pyrolyser or ferromagnetic wires with defined Curie temperatures, is useful in detecting certain building blocks, including polysaccharide-like substances, aromatic units, cyclopentenone units, *N*-containing units and aliphatic units in the HOM network, thus giving some evidence of the structure, origin, genesis, degree of decomposition and humification (condensation) of the polymer under study. As an example, pyrograms of marine samples are rich in protein products derived from phytoplankton, but are free from the lignin-derived guaiacyl (4-hydroxy-3-methoxyphenol) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) compounds which are common in terristrial and freshwater samples. Although there are many sources of typical fragments in the pyrolysate, it is generally accepted that furfural, levoglucosan (and other anhydrosugars), pyranones and acetic acid originate from carbohydrates, methoxyphenols from wood and lignin, ammonia, acetonitrile and pyrrole from proteins, while alkanes, alkylbenzenes (-naphthalenes, -phenanthrenes, etc.) and fatty acids originate from fossil fuels and biomass. Pyrograms of soil- and sediment-derived HOM are mainly characterized by the classical *n*-alkane/*n*-alk-l-ene/*n*-α,ω-alkadiene triplet series.

They may originate from esters, the saturated alkanes originating from decarboxylation of the fatty acid moiety, and the olefins from the alcohol moiety as a result of β-scission. Another source may be alkylbenzenes with long side chains.

Generally, pyrograms are very complex, composed of several hundreds of compounds. **Figure 1** shows two extracted ion chromatograms used to trace thiophenes and benzenediols in the complex pyrogram. The (anthropogenic) fulvic acid under study was isolated from a coal wastewater pond, where it was formed spontaneously from coal wastewater components. High concentrations of phenols and benzenediols as well as of heterocyclic compounds are striking in comparison with pyrograms of HOM isolated from natural sources. The comparison between these patterns in the fulvic acid isolated from the coal wastewater, in the HOM of the associated sediment on the bottom of the pond, in the organic solvent extract of the sediment (obtained by means of accelerated solvent extraction or ASE) and the pollutant pattern in the wastewater allows conclusions to be drawn on the humification process and on the pathways of contaminants present in the coal wastewater.

Reproducibility of the pyrograms is mainly determined by the steps in sample preparation, pyrolysis and pyrolysate transfer to the GC column rather than by the pyrolysate analysis by GC-MS (AED). Prior to pyrolysis, the HOM sample is usually subjected to an intense degreasing procedure, e.g. by Folch's chloroform–methanol (2 : 1) mixture to avoid ambiguous results with regard to the source of the pyrolysate. The extract from exhaustive solvent extraction contains the same compounds which are released during thermal desorption at subpyrolysis temperatures (e.g. 300°C). Both methods reveal contaminants attached to the HOM rather than covalently bound. To obtain a more homogeneous material and to simplify the pyrogram, a combination of pyrolysis and chemical modifications may be used: for example, acid hydrolysis removes carbohydrates and proteins/peptides, whereas the more resistant hydrocarbon fraction remains unchanged.

In general, pyrograms of HOM reveal overwhelmingly nonpolar hydrocarbons and less polar

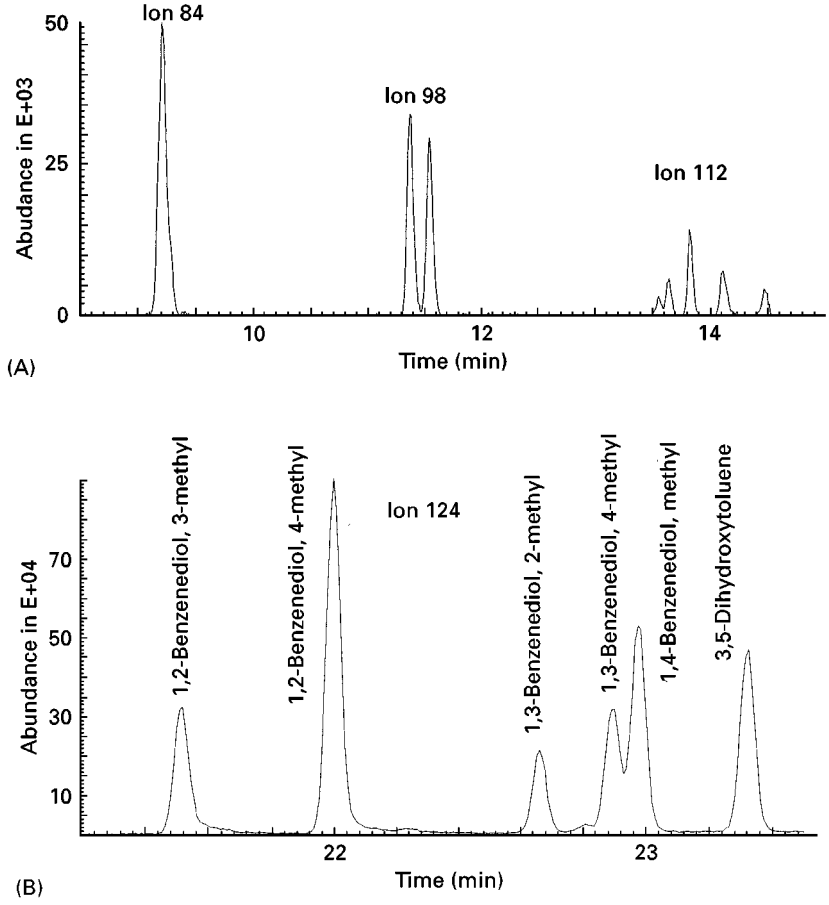

Figure 1 (A) Thiophene and (B) benzenediol pattern of an aquatic humic acid isolated from coal wastewater on an inert HP-5 capillary column.

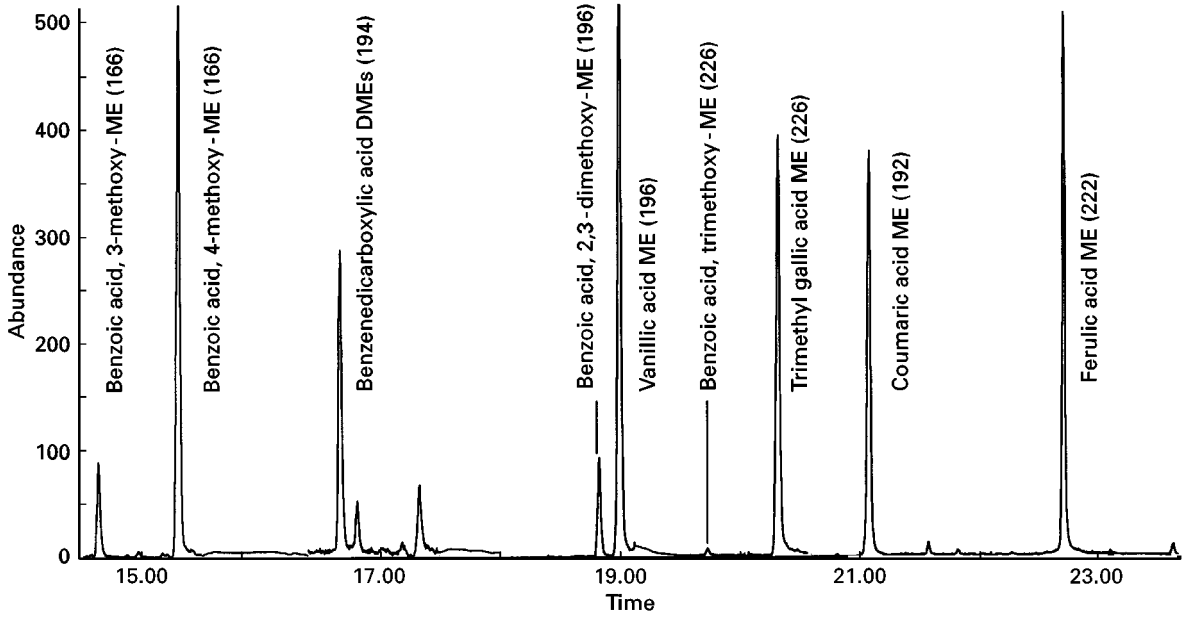

Figure 2 Aromatic methyl esters (ME) obtained in TMAH-assisted *in situ* methylation of a humic acid isolated from peat. Selected ion traces are given in parentheses.

heterocyclic compounds (e.g. benzofuranes, chinolines, benzothiophenes, etc.). Evidently, there are large discrepancies between NMR results and common titration results on the one hand, which indicate high carboxyl and hydroxyl group contents, and the basically nonpolar pyrolysates on the other hand. This finding is due to pitfalls occurring during pyrolysis, like the decarboxylation of fatty acids to give alkanes and the formation of polar, less volatile products inaccessible to GC (see below). Some efforts have been made to overcome these drawbacks. The coupling of pyrolysis with the 'soft' field ionization MS to give overwhelmingly molecular ions of thermal degradation products, including polar and higher molecular ones, turned out to be very useful in rapid profiling of HOM. The significance of this strategy can be further enhanced by using high resolution MS. The hyphenated technique thermogravimetry-MS, which allows running experiments in a time-resolved manner, can give further information on the binding state of the compounds released from the matrix. Another approach is to produce appropriate derivatives to preserve the analytical integrity of HOM in a better way.

Thermochemolysis with TMAH

Challinor was the first to introduce *in situ* methylation using a methanolic solution of tetramethylammonium hydroxide (TMAH) with biopolymers to convert polar carboxylic and hydroxyl groups to less polar and GC-accessible methyl derivatives so as to avoid decarboxylation and dehydration. Methodological studies revealed that simultaneous pyrolysis/methylation (SPM) is not a pyrolysis but a thermally assisted chemolysis. This saponification/esterification reaction can be performed at subpyrolysis temperatures in both an online and offline (batch) approach, the latter including flushing of the thermochemolysis products into an organic solvent spiked with internal standards.

In contrast to conventional pyrolysis, some compound classes of diagnostic value can be detected by means of the SPM approach:

1. Aromatic acids, including mono-, di- and trimethoxybenzenecarboxylic acid methyl esters, benzenedicarboxylic acid dimethyl esters, mono- and dimethoxybenzenepropenoic acid methyl esters (**Figure 2**). The presence of trimethylgallic acid (3,4,5-trimethoxybenzoic acid) is of special diagnostic value, because it indicates the final step in the oxidation of side chains in lignin units[1]. To verify the ambiguous origin of methoxy compounds (real methoxy moieties or resulting from originally free hydroxyls), deuterated TMAH can be used[2]. Another approach is the application of

[1] The 3,4,5-trimethoxybenzoic acid methyl ester may also be derived from syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid).

[2] Analogously, to investigate the role of methanolysis, the reagent TMAH/CD_3OH may be used.

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tetrabutylammonium hydroxide, giving rise to *O*-butyl ethers only from phenolic groups, whereas aliphatic hydroxyls do not undergo a reaction with the weaker acidic reagent.

2. Long chain fatty acid methyl esters (FAMEs). A pronounced even-over-odd discrimination constitutes a strong evidence of biogenic input, while uniform FAME patterns indicate anthropogenic origins. Iso- and anteiso FAMEs (frequently C_{15} and C_{17}) are related to Gram-positive bacterial activities, whereas *cis*-vaccenic acid ($C_{18:1}$, double bond in $\Delta 11$ position) is related to Gram-negative bacteria.
3. The homologous series of α,ω-dicarboxylic acid methyl esters (DME). The α,ω-alkanedioic acids are of distinctive diagnostic value because they are thought to act as bridges in the HOM network and thus are considered to be markers for the cross-linking ratio. However, other pathways cannot be ruled out: the C_9-DME is widely distributed in both aquatic and terrestrial HOM of natural origin. It may originate from fatty acids with double bonds in $\Delta 9$ position, e.g. oleic acid, which have undergone oxidation. Analogously, the C_{11}-DME is common in pyrolysates of bacteria, the lipids of which contain distinctive *cis*-vaccenic acid moieties.
4. The pristenes refer to chemically bound phytol and tocopherols.
5. 1-Methoxyalkanes formed by thermochemolysis represent mainly alkanols bound by ester groups.

SPM at 'mild' temperatures followed immediately by thermal treatment under more severe conditions can be used to estimate the share of ester, ether and C–C bonds in the HOM polymeric network. Pyrograms of HOM of both aquatic and terrestrial origin reveal a pronounced FAME pattern under mild conditions at 500°C, but do not reveal any alkanes. SPM at 750°C after the treatment at 500°C gives typical *n*-alkane patterns originating either from ether linkages or from alkylbenzenes. These findings may be attributed to the fact that ester bonds are cleaved at 500°C, whereas the more stable C–O–C and C–C bonds are resistant to decomposition at this 'mild' temperature.

Limitations of Pyrolysis in Structure Elucidation of HOM

Generally, pyrolysis work is aimed at obtaining a maximum yield of pyrolysis products to be analysed by means of GC and at ensuring that these volatile products reflect the structure of the polymer from which they are generated as truly as possible[3].

It was not until the mid 1990s that *Kopinke* carried out the first quantification experiments with HOM pyrolysis using a two-detector technique (MS to identify the pyrolysate, flame ionization detector (FID) to quantify organic carbon). Before that time, quantification was done in a semiquantitative way at best (+ + + most abundant, + + less abundant, etc.). Calibration can be done offline using on-column injection of calibration mixtures, or can be performed by vaporizing internal standards in the hot pyrolysis interface during equilibration just before the beginning of the pyrolysis process. Three fractions can be distinguished in the pyrolysate, the sum of which amounts to almost 100% of the original HOM organic carbon:

1. A solid residue, which consists almost exclusively of carbon, due to charring reactions resulting from the elimination of functional groups and additional cross-linking. This residue is about 30% of the original organic carbon content, largely independent of the kind of HOM studied. Thus, considering the fact that HOM consists of about 40–50% organic carbon, over 50% of the HOM's carbon, which is expected to carry substantial structural information, is lost on pyrolysis. Unfortunately, this residue cannot be significantly reduced by increasing the pyrolysis temperature, and it is also in the same order of magnitude when pyrolysing under in-source vacuum conditions. The latter finding indicates that the 'coke' formation is a solid-phase reaction rather than a transport-limited step. When pyrolysing soils, the tendency to form solid carbonaceous residue is correlated to organic carbon content.
2. Compounds in the pyrolysate with little structural significance. The remaining carbon is detected mainly as nonspecific light gases, including carbon dioxide, carbon monoxide and methane eluting at the beginning of the chromatogram. Although carbon dioxide is assumed to be a rough measure of decarboxylation and methane a rough measure of methyl substitution, etc., all these light gases are of minor diagnostic relevance to the HOM structure.
3. Compounds with supposed structural significance ($C > 4$, assumed arbitrarily), comprising about

[3] Losses due to the limitations of the analytical system are outside this consideration. They may be circumvented by pyrolysis inside a GC precolumn; all volatile pyrolysates are completely transferred to the column.

6–10% of the organic carbon. A slight increase in the yield of structure-related compounds can be observed when turning from the conventional pyrolysis to SPM (15% at best).

However, the situation gets even worse when considering the real significance of the compounds in group 3 above. Whereas long aliphatic chains definitely originate from the HOM skeleton, aromatic and cyclic compounds may undergo unwanted thermal modifications, resulting in their misinterpretation, as already discussed above with alkanes/fatty acids. Further, the occurrence of furanes in the pyrolysate does not necessarily mean that furane moieties are present in the HOM backbone. It can also be related to thermal rearrangements and reactions originating from polysaccharide-related units (e.g. xylans, cellulose from terrestrial plants) or OH-substituted carbon chains with at least four carbons. Hence, if pyrolysis products are considered to be building blocks, cellulose should be made up of furans, pyranones, anhydrosugars, etc. Similar pitfalls occur with alkylbenzenes. They can be formed in the cracking of triglycerides and fatty acids (catalytically stimulated, e.g. by elemental sulfur). Most recent findings also indicate the formation of alkylbenzenes via a metal-catalysed alkylation of aromatic hydrocarbons, the cations being complexed in the HOM network. Therefore, structural models based on conventional pyrolysis studies are largely biased.

SPM is considered to be less error-prone. However, this approach has not been scrutinized as closely as conventional approach up until now. Saiz-Jimenez, who pioneered the SPM approach, found that model phenolic acids underwent decarboxylation reactions. Most recent results indicate that the occurrence of benzene carboxylic acids in the pyrogram does not necessarily indicate their presence in the HOM backbone: lignin models free of carboxylic groups may yield MEs of benzenecarboxylic acids during SPM. On the other hand, decarboxylation reactions and isomerization of unsaturated fatty acids have not been under intense study yet, but presumably they cannot be completely excluded. Hydroxyl- and carbonyl-containing resin acid MEs have been shown to undergo side reactions with the reagent, resulting in the formation of nitrogen-containing derivatives. Likewise, aldehydes have been recently shown to undergo a Cannizarro-like reaction, in which the products are methylated to esters and ethers. In addition to that, the quantity of FAMEs released in 500°C thermochemolysis surpasses by far the quantity of hydrocarbons (including alkanes, alkenes and alkylbenzenes) released in conventional pyrolysis, pointing to further aliphatic precursors. Moreover, the FAME pattern does not necessarily coincide with the alkane pattern.

Apart from the ambiguous origin of markers in the pyrograms of HOM, these markers cannot be assigned to defined biological precursors by themselves. As an example, *n*-alkanes can be derived from anthropogenic sources (fuels), from direct input of biosynthesis (higher plants), from reduction of fatty alcohols (bacteria, higher plants), from decarboxylation of almost ubiquitous fatty acids and from depolymerization of aliphatic biopolymers. To assign these sources, isotope ratio GC-MS is very useful.

Controlled Wet Chemical Degradation

The ultimate aim of this approach (mostly conducted in an offline mode), elucidation of the HOM structure on the basis of degradation products, is similar to that of pyrolysis. As with HOM pyrolysis, large quantities of nondiscriminated building blocks are expected from an ideal chemical degradation method in a mechanistically predictable manner[4]. Common methods to obtain defined degradation products include mainly traditional approaches, such as:

1. Reductive degradation, e.g. using catalytic hydrogenation or alkali metals such as sodium or sodium amalgam in liquid ammonia, or lithium in liquid ethylamine. (The free or solvated electrons are provided in metallic reductions by the conversion of metal atoms to their cations.) In all cases, the carbon skeleton of aliphatic substructures is claimed to remain intact, whereas bonds between carbon and heteroatoms (in particular oxygen) are cleaved. A reagent with reactivity slightly higher than that in catalytic hydrogenolysis is iodotrimethylsilane, capable of cleaving ether and ester bonds. **Figure 3** shows the fatty acid pattern of a terrestrial HOM isolated from the sediment at the bottom of a coal wastewater pond after this treatment followed by methylation. The fatty acid pattern reflects both natural sources, expressed by the even-over-odd discrimination in the C_{14}–C_{18} range, and the presence of bacterial iso- and anteiso-acids and anthropogenic sources, expressed by very long chain acids and a less pronounced even-over-odd discrimination at higher carbon numbers.
2. Oxidative and hydrolytic degradation. Common oxidative reagents include cupric oxide, ruthenium tetroxide and potassium permanganate. The

[4] The derivatization of HOM has also been used for subsequent analysis by NMR or infrared spectroscopy to obtain well-resolved spectra, e.g. by silylation of groups with acidic protons.

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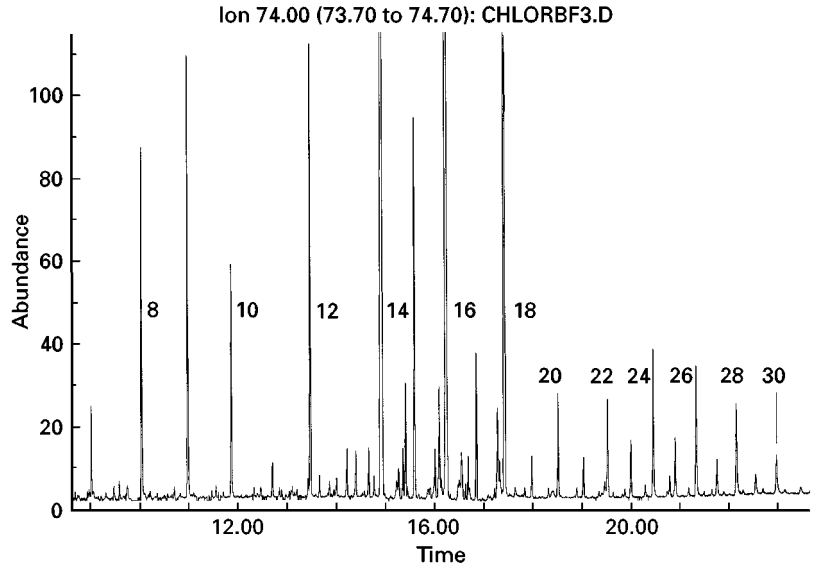

Figure 3 Fatty acid pattern (as methyl esters) of an HOM isolated from a sediment associated with coal wastewater, obtained after iodotrimethylsilane cleavage (using chlorotrimethylsilane–sodium iodide) and boron trifluoride catalysed methylation. Numbers indicate the carbon chain length of the homomorphic fatty acid.

hydrolysis is mainly catalysed either by sodium hydroxide or sulfuric/hydrochloric acid[5]. Another direction includes mild and selective enzymatic hydrolysis. As with thermochemolysis with TMAH, these approaches revealed products, including benzene mono-, di-, tri- and tetracarboxylic acids, furane di- and tricarboxylic acids, aliphatic mono-, di- and tribasic acids and (di-, tri- and tetracarboxyphenyl) glyoxylic acids, all of them preferably subjected to GC as MEs or trimethylsilyl ethers. CuO is known to oxidize the lignin macromolecule (cleavage of the β O-4 ether bond, as with TMAH thermochemolysis) and produces a series of *p*-hydroxyphenyl, guaiacyl and syringyl compounds.

3. Transesterification, e.g. using boron trifluoride–methanol. The yields from this milder approach are quite low, but the formation of by-products and the destruction of labile structures are minimized.

As with pyrolysis, chemical degradation may be full of pitfalls. Hexose sugars are known to dehydrate to hydroxymethylfurfural; pentoses give furfural in strong acids at elevated temperatures. Moreover, melanoidins may be formed from sugars in the presence of amino acids leading to enhanced (apparent) aromaticity. Diols can be oxidized, including ring rupture, to form low molecular weight aliphatic acids. Gallic acid can be decarboxylated on heating in water, and so forth. Although the loss in weight of the HOM on chemical degradation gives yields between 3 and 30%[6] of the mass of the starting polymer, the yields of products accessible to GC are significantly lower, in the range of 0.5–3%. A large percentage (about 25%) of the total organic carbon is lost as CO_2 by $KMnO_4$ oxidation. FTIR spectra give strong evidence that permethylation cannot remove all acidic protons. Thus, extrapolation to the macromolecular skeleton is quite biased.

Selective chemical degradation has also been used in a more restricted way to elucidate the fate of organic pollutants and metabolites in soils/sediments, which cannot be released by means of organic solvent extraction. This results in a better understanding of their incorporation into the macromolecular HOM and their remobilization potential. Pioneering work has been done by *Michaelis*, who cleaved bound polycyclic aromatic hydrocarbon (PAH) metabolites such as hydroxynaphthoic acids from the macromolecular HOM network using boron trichloride (capable of degrading ester and ether bonds) as well as by alkaline hydrolysis, and degraded phenolic entities from the HOM matrix by rhodium on charcoal

[5] Hydrolysis proceeds faster and more efficiently if the HOM is dissolved in the hydrolytic reagent; thus, alkaline hydrolysis should be used with humic acids, which by definition are insoluble in acids.

[6] Empirical findings suggest that a methylation procedure prior to degradation is beneficial towards yields of oxidative products of HOM.

hydrogenolysis. The type of bonding and linkage of pollutants/metabolites can be better elucidated using isotope-labelled $Na^{18}OH$, because only the carboxylic entity of the ester carries the heavier oxygen isotope, thus referring to products attached to the HOM matrix by ester bonds.

Although wet chemical degradation techniques as well as pyrolysis techniques involve some pitfalls and limitations, they can contribute to elucidation of the fundamentals of the diagenesis of organic compounds in soil and water. ^{13}C NMR results yield strong evidence that a large percentage (in general, about 60% in case of the HOM under study) of methyl groups is attached to hydrogen-free paraffinic carbon. These findings cannot be explained by building blocks consisting of alkanes or alkylbenzenes. Steranes and hopanes, considered to be the end products of a complex web of diagenetic reactions starting from functionalized bacteriohopanepolyols and serving as biomarkers in ancient sediments and petroleum, are assumed to account for that. Indeed, pentacyclic terpanes with 32–35 carbon atoms, confirmed by tracing $m/z = 191$ in the GC-MS mode, can be detected using both chemical and thermal degradation.

Future Developments

Conventional and TMAH pyrolysis, as well as controlled chemical degradation methods, applied together in combination with highly efficient GC-MS can give insight to the building blocks and the linkages between them in the polymeric HOM network. However, analytical pyrolysis has a significantly higher potential in revealing the chemical nature of simpler polymers, e.g. polyethylene and polystyrene, which give no or minor solid residue. Traditionally performed pyrolysis work with HOM conceals much significant information on the chemical nature of HOM by thermal degradation of functional groups and thermal rearrangements. Thermochemolysis with TMAH gives rise to complementary and more independent reactions in comparison with conventional pyrolysis. Not much attention has been paid to improved specificity of bond cleavage in the structures. It will be important to understand the mechanisms of the cleavages, and to be able to relate the products identified to possible structures in the parent macromolecule.

See also: **II/Chromtography: Gas:** Derivatization; Detectors: Mass Spectrometry; Pyrolysis Gas Chromatography.

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Liquid Chromatography

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Humic substances are complex mixtures of compounds and in order adequately to elucidate and characterize the properties and reactions of humic substances, separation techniques are an absolute requirement. Initial work in this area employed low pressure liquid chromatography (LC) with extensive use of size exclusion chromatography (SEC). The advent and acceptance of high performance liquid